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### Nuclear S-nitrosylation impacts tissue regeneration in zebrafish

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1   **Title: Nuclear S-nitrosylation impacts tissue regeneration in zebrafish**

2   **Authors:** Gianfranco Matrone 1,2\*, Sung Yun Jung 3,4, Jong Min Choi 3, Antrix Jain 3,  
3   Hon-Chiu Eastwood Leung 5, Kimal Rajapakshe 5, Cristian Coarfa 5, Julie Rodor 1, Martin  
4   Denvir 1, Andrew H Baker 1 and John P Cooke 2.

5   **Affiliations:**

- 6   1. British Heart Foundation Centre for Cardiovascular Science, Queen's Medical Research  
7   Institute, The University of Edinburgh, 47 Little France Cres, EH16 4TJ, Edinburgh, UK.  
8   2. Center for Cardiovascular Regeneration, Department of Cardiovascular Sciences, Houston  
9   Methodist Research Institute, Houston, TX, 77030, USA  
10   3. Mass Spectrometry Proteomics Core, Baylor College of Medicine, Houston, TX, USA.  
11   4. Department of Biochemistry and Molecular Biology, Baylor College of Medicine,  
12   Houston, TX, 77030, USA.  
13   5. Department of Molecular & Cell Biology, Baylor College of Medicine, Houston, TX,  
14   77030, USA.

15

16   \* Corresponding author

17   **Correspondence to:** Gianfranco Matrone, Centre for Cardiovascular Science, Room E3.07,  
18   Queen's Medical Research Institute, University of Edinburgh, 47 Little France Crescent,  
19   Edinburgh EH16 4TJ, United Kingdom. Email: [gianfranco.matrone@ed.ac.uk](mailto:gianfranco.matrone@ed.ac.uk) , Tel: +44  
20   (0)131 242 9334;

21   **Key words:** S-nitrosylation, nuclear protein, Kdmla, zebrafish, regeneration.

22

23    **Abstract**

24    Despite the importance of nitric oxide signaling in multiple biological processes, its role in  
25    tissue regeneration remains largely unexplored. Here, we provide evidence that inducible  
26    nitric oxide synthase (iNos) translocates to the nucleus during zebrafish tailfin regeneration  
27    and is associated with alterations in the nuclear S-nitrosylated proteome. iNos inhibitors or  
28    nitric oxide scavengers reduce protein S-nitrosylation and impair tailfin regeneration. Liquid  
29    chromatography/tandem mass spectrometry reveals an increase of up to 11-fold in the  
30    number of S-nitrosylated proteins during regeneration. Among these, Kdm1a, a well-known  
31    epigenetic modifier, is S-nitrosylated on Cys334. This alters Kdm1a binding to the CoRest  
32    complex, thus impairing its H3K4 demethylase activity, which is a response specific to the  
33    endothelial compartment. Rescue experiments show S-nitrosylation is essential for tailfin  
34    regeneration, and we identify downstream endothelial targets of Kdm1a S-nitrosylation. In  
35    this work, we define S-nitrosylation as an essential post-translational modification in tissue  
36    regeneration.

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## 48    **Introduction**

49    Complete regrowth of functional tissue is a highly desirable, but mostly unachieved,  
50    therapeutic target in the tissue loss associated with many human diseases. Innate immune  
51    activation is an early response to tissue stress and injury <sup>1</sup>. In lower vertebrates this is  
52    typically followed by functional tissue regeneration while in higher vertebrates there is  
53    normally fibrous scar formation <sup>2</sup>. Although several molecular pathways have been  
54    implicated in tissue regeneration, the mechanisms underlying this process are still not clearly  
55    understood <sup>3</sup>.

56    The interaction between pattern recognition receptors (PRRs) and damage-associated  
57    molecular patterns (DAMPs) during and after an injury activates molecular pathways and  
58    transcriptional factors that regulate the expression of a plethora of genes. We have previously  
59    shown that this inflammatory signaling causes global changes in the expression and post-  
60    translational modifications (PTM) of epigenetic modifiers favoring an open chromatin  
61    configuration and cellular plasticity <sup>4,5</sup>. It was also found that inducible nitric oxide synthase  
62    (iNos) translocates to the nucleus to bind and S-nitrosylate the polycomb and NuRD  
63    complexes during trans-differentiation of fibroblasts into endothelial cells <sup>6</sup>. The effect of cell  
64    autonomous innate immune signaling to increase DNA accessibility and thereby to facilitate  
65    nuclear reprogramming of cell fate is termed transflammation <sup>7</sup>. The role of this phenomenon  
66    in tissue regeneration remains unexplored.

67    Accordingly, here we investigated the role of the innate immune effector iNos and S-  
68    nitrosylation of nuclear proteins in zebrafish tailfin regeneration, an ideal model to study  
69    appendage regeneration <sup>8</sup>. We found that *inos* translocates from the cytoplasm to the nucleus  
70    in the regenerating tailfin and this is associated with an increase in S-nitrosylation of over  
71    500 different nuclear proteins. Of these, we demonstrated a strong link between Kdm1a S-

72 nitrosylation and histone demethylase activity during tailfin regeneration, specifically in  
73 endothelial cells, the main target where S-nitrosylation mostly occurs and where the S-  
74 nitrosylated form of Kdm1a promotes the expression of proangiogenic genes. Here, we show  
75 the essential role of the S-nitrosylation of nuclear proteins in tissue regeneration.

76

## 77 **Results**

78 *Nos2* translocates from the cytoplasm to the nucleus and triggers protein S-nitrosylation  
79 during tailfin regeneration.

80 Compared to mammals that have three *nos* genes (neuronal, or *nos1*; inducible, or *nos2*; and  
81 endothelial, or *nos3*), the zebrafish genome has only *nos1* and two *nos2* genes (*nos2a* and  
82 *nos2b*). Activation of the innate immune system transcription factor Nf-kb following injury  
83 triggers the translocation of the kb subunit into the nucleus which in turn promotes activation  
84 of a panoply of genes, including *inos*<sup>9</sup>. The activation of Nf-kb after tailfin amputation was  
85 measured as GFP signal in the *Tg(nfkb:EGFP)<sup>nc1</sup>* zebrafish (**Fig. S1A-B**). Real time PCR for  
86 *nos* genes measured in the adult zebrafish (*Danio rerio*) tailfin uninjured (baseline) and at 3,  
87 5 and 10 days post-amputation (dpa) revealed a significant increase of *nos2b* expression and  
88 a slight increase in *nos1* at 3 and 5 dpa (**Fig. 1A**). Increased *nos2* expression during  
89 regeneration was confirmed by western blotting (WB) (**Fig. 1B**). To confirm the role of Nf-  
90 kb on the upregulation of *nos2*, adult zebrafish were injected with the Nf-kb inhibitor Bay11-  
91 7082 30 mM, which resulted in a reduced expression of *nos2b* in the injured tailfin (**Fig.**  
92 **S1C**) at 3 and 5 dpa compared to injured control. While iNos has been considered  
93 predominantly cytosolic, it has also been localized to other cellular compartments, including  
94 the nucleus<sup>6</sup>. Analysis of *Nos2* compartmentalization in the uninjured tailfin confirmed a  
95 predominantly cytoplasmic distribution of this protein (**Fig. 1C**). However, at 3 dpa *Nos2*

96 was predominantly in the nucleus, with equal distribution between nucleus and cytoplasm at  
 97 5 dpa, returning predominantly to the cytoplasm by 10 dpa, similar to uninjured controls.  
 98 Thus, immediately after injury, Nos2 translocates to the nucleus and, during later stages of  
 99 repair, shifts back to the cytoplasm. This key series of observations gave rise to our principal  
 100 hypothesis, namely, that changes in the distribution of Nos2 during regeneration mirror and  
 101 drive changes in S-nitrosylation of nuclear proteins.  
 102 To address this, we collected newly formed tissue at the wound edge for extraction of nuclear  
 103 protein after amputation of adult zebrafish tailfins. Separation of nuclear proteins was  
 104 confirmed by western blotting to detect nuclear and cytoplasmic markers (**Fig. S1D**). The  
 105 nuclear fractions were treated with iodoacetyl Tandem Mass Tags (iodoTMT) to label S-  
 106 nitrosylated proteins, and were subsequently identified using an anti-TMT antibody. Western  
 107 blotting revealed an increase in the number and intensity of protein bands in the regenerating  
 108 tissue compared to control tissue, suggesting an S-nitrosylation switch in nuclear proteins  
 109 (**Fig. 1D**). When zebrafish were injected in the retro-orbital vein with increasing  
 110 concentrations of the Nos inhibitor N( $\omega$ )-nitro-L-arginine methyl ester (L-NAME) or the NO  
 111 scavenger 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), the levels of  
 112 protein S-nitrosylation were reduced (**Fig. 1E**), whereas treatment with increasing  
 113 concentrations of the NO donor S-Nitroso-N-acetyl-DL-penicillamine (SNAP) increased  
 114 protein S-nitrosylation (**Fig. 1E**). L-NAME and PTIO significantly reduced tailfin  
 115 regeneration compared to control or SNAP treated groups at 3 and 7 dpa (**Fig. 1F and S2A**).  
 116 As L-NAME inhibits all Nos isoenzymes, in order to assess the specific role of iNos we  
 117 treated the tailfin regeneration model with the iNos selective inhibitor 1400W<sup>10</sup>. Treatment  
 118 with 1400W 50 mM reduced tailfin regeneration in a manner similar to L-NAME, suggesting  
 119 that Nos2 is the main Nos isoenzyme involved in the effect observed by L-NAME (**Fig.**

**S2A**). Overall, these results indicate that Nos2 and NO are necessary for tailfin regeneration and that protein S-nitrosylation plays a key role in this process.

Analysis and screening of S-nitroso-proteome revealed an increase in S-nitrosylated nuclear proteins during tailfin regeneration.

To identify nuclear proteins that were S-nitrosylated during regeneration, we performed liquid chromatography and tandem mass-spectrometry (LC/MS/MS) (**Fig. 2A**) on protein extracts from the tailfin wound edge, excised at 3, 5 and 10 dpa. Hierarchical clustering showed a striking increase in the number of nuclear S-nitrosylated proteins during regeneration, in particular at 5 dpa (**Figs. 2B and S3**). We found that the number of nuclear S-nitrosylated proteins increased from 31 in uninjured to 199, 351 and 264 at 3, 5 and 10 dpa, respectively (**Fig. 2C**). Taking account of the fact that some proteins changed their S-nitrosylation state on more than one cysteine residue of that protein, the actual total number of S-nitrosylated peptides increased from 31 in uninjured to 332, 566 and 450 at 3, 5 and 10 dpa, respectively (**Fig. S3A**). While a few proteins were S-nitrosylated throughout these time-points, suggesting constitutive S-nitrosylation, the analysis revealed that the majority of the proteins were modified uniquely at a specific time-point. These data strongly suggest a dynamic choreography of S-nitrosylation throughout the regeneration process. Indeed, an analysis of differential enrichment revealed that proteins of many different pathways are S-nitrosylated specifically during regeneration (**Fig. 2D**). Some of these are known to be implicated in developmental and wound healing processes, such as the epithelial–mesenchymal transition (EMT) pathway<sup>11</sup> and the Hedgehog pathway<sup>12</sup>. From the protein list (**Dataset S1**), we selected 31 candidates, encompassing epigenetic modifiers and transcription factors for which transient S-nitrosylation have not been previously reported and

144 that have a human ortholog (**Table S1**). We proceeded to analyse these by deep mass-  
 145 spectrometry, focusing initially on Kdm1A, also known as Lsd1, Kiaa061 or Aof2  
 146 (UniProtKB - F6NIA2).

147

148 S-nitrosylation of Kdm1a is associated with a reduced binding to the CoREST complex and  
 149 impaired demethylase activity on H3K4 during tailfin regeneration.

150 Kdm1a was the first histone lysine-demethylase to be described<sup>13</sup>, where an amine oxidase  
 151 domain mediates its FAD-dependent demethylase activity<sup>14</sup>. Kdm1a participates in gene  
 152 repression as part of the CoREST (co-repressor for element-1-silencing transcription factor)<sup>15</sup>  
 153 and NuRD (nucleosome remodeling and histone deacetylation)<sup>16</sup> co-repressor complexes  
 154 mediating the demethylation of H3K4me1/me2. It also participates in gene activation in  
 155 androgen receptor (Ar)-driven expression programs through demethylation of repressor  
 156 marks H3K9me1/me2<sup>17,18</sup>. More recently, it has been demonstrated that Kdm1a is also able  
 157 to demethylate lysine residues at several non-histone substrates, such as p53<sup>19</sup>, Dnmt1 (DNA  
 158 (cytosine-5)-methyltransferase 1)<sup>20</sup> and E2F1<sup>21</sup>.

159 Mass-spec revealed that Kdm1a, although detectable in the nucleus at all three time-points,  
 160 becomes S-nitrosylated on Cysteine 334 (Cys334) at 3 and 5 dpa (**Fig. 3A-B**). The increase  
 161 of Kdm1a S-nitrosylation (S-NO) during tailfin regeneration was confirmed by western  
 162 blotting for S-NO on samples previously immunoprecipitated for Kdm1a (**Fig. 3C**). Zebrafish  
 163 Kdm1a protein has 85% identity with the corresponding human gene with the Cys334  
 164 corresponding to Cys360 in the human ortholog (**Fig. S4A**). Intriguingly, the sequence of 60  
 165 amino acids (aa) from 50 upstream to 9 downstream of the Cys334 retains 100% identity with  
 166 the human ortholog. Human KDM1a crystal structure, obtained from Protein Data Bank  
 167 deposited by Tan *et al.*<sup>22</sup>, is shown in **Fig. S4B**.



168 The selectivity of Kdm1a for its main targets, H3K4 and H3K9, depends on its interaction  
169 with specific protein co-factors, including CoREST, NuRD, and Ar. PTM can modulate these  
170 interactions. Therefore, first we analysed these co-factors and their complexes in injured and  
171 uninjured tailfins. Expression of CoREST and NuRD were unchanged in injured and  
172 uninjured tailfin (**Fig. S5**). The expression of *ar* was extremely low and was not different in  
173 injured versus uninjured tailfin (**Fig. S5**). Having established the patterns of expression of  
174 these co-factors, and since *ar* is associated with the modulation of H3K9, we decided to focus  
175 on H3K4 which is known to be linked to CoREST and NuRD.

176 Hence, focusing on NuRD and CoREST, we tested the hypothesis that S-nitrosylation of  
177 Cys334 affects Kdm1a binding affinity to these complexes. To do this, we performed co-  
178 immunoprecipitation with Kdm1a antibody (**Fig. 3D**), followed by WB for the members of  
179 the CoREST (Rcor1 and Hdac1) (**Fig. 3E**) and NuRD (Rbbp4 and Chd4) (**Fig. 3F**)  
180 complexes. While the binding of NuRD complex was similar during regeneration compared  
181 to control, the binding to the CoREST complex was reduced.

182 To further assess the role of Kdm1a in the adult zebrafish, knockdown (KD) was achieved by  
183 injection of morpholino (vivo-Mo) in the retro-orbital vein. The KD efficiency as well as  
184 delivery to the tailfin was assessed by western blotting for Kdm1a (**Fig. 4A**). We saw clear  
185 evidence that *kdm1a* KD by vivo-Mo injection impaired tailfin regeneration compared to  
186 controls (**Fig. 4B-C**). A dynamic and choreographed regulation of *kdm1a* expression and  
187 activity therefore appears to be essential for tissue regeneration. These data support the role  
188 of Kdm1a as a key regulator of regeneration, likely through modulation of chromatin  
189 modifications.

190 During tailfin regeneration we observed a significantly reduced demethylase activity of  
191 Kdm1a in the regenerating tailfin associated with increased levels of Kdm1a S-nitrosylation

(**Fig. 3B**). This reduced Kdm1a activity was similar to that observed in zebrafish *kdm1a* KD (**Fig. S6**).

Indeed, at the same time that Kdm1a activity was reduced, there was a corresponding reduction in unmethylated H3K4 (**Fig. 4D-E**). By contrast, there was an increase in H3K4me1/me2, normally associated with active gene transcription (**Fig. 4D-E**). H3K4me3 showed a pattern similar to H3K4me2. However, it is known that Kdm1a is unable to demethylate H3K4me3, according to the chemical nature of the amine oxidation reaction catalyzed by flavin-containing amine oxidases, thus precluding H3K4me3 as a substrate<sup>13</sup>.

In regenerating tailfins, the blastema, a pool of proliferative progenitor cells, is located at the distal region of the tailfin while cells in the proximal region undergo differentiation. To localise the region in which Kdm1a S-nitrosylation is most important, we analysed the expression of *kdm1a* gene in the distal (blastema) and proximal regions (differentiating zone) of regenerating tailfins at 3 and 5 dpa. Compared to uninjured tailfins, *kdm1a* expression was significantly lower in the blastema and only slightly reduced in the proximal differentiating zone (**Fig. S7A**). We also took advantage of a publicly available single cell(sc) RNA-seq dataset obtained in the regenerating tailfin<sup>23</sup> in which *kdm1a* expression was detected, at low levels, with a distinct expression pattern observed in specific cell types (**Fig. S7B**). For example, compared to preinjury, the *kdm1a* expression was reduced in mucosal-like cells at 1 and 2 dpa followed by an increase at 4 dpa, it was increased in mesenchymal cells and it was not expressed in hematopoietic cells during the regeneration. Overall, this data provides further evidence that modulation of S-nitrosylation, as well as modulation of gene expression, is a key mechanism regulating the functionality of Kdm1a during tailfin regeneration.

215 *Kdm1a* is S-nitrosylated preferentially in the endothelial cells of the regenerating tailfin.

216 Next, we identified S-nitrosylation associated with specific cell type. The formation of new  
 217 blood vessels is a crucial process during wound healing and tissue repair, similar to  
 218 embryogenesis and early growth. During tailfin regeneration, endothelial cells within the  
 219 growing blood vessels sprout and invade adjacent avascular areas. By using the  
 220 *Tg(fli1:EGFP)<sup>y1</sup>* zebrafish line, where endothelial cells (EC) fluoresce green (GFP<sup>+</sup> cells), we  
 221 detected the formation of new blood vessels in the regenerating tailfin at 2 dpa that become a  
 222 dense vascular plexus by 3 dpa (**Fig. 5A**) consistent with previous observations<sup>24</sup>. We  
 223 isolated EC from these tailfins and performed FACS analysis. We found an increase in the  
 224 percentage of EC (GFP<sup>+</sup>) from  $4.3 \pm 0.3$  % to  $6.1 \pm 0.4$  %, in the uninjured versus injured  
 225 tailfin, respectively (**Fig. 5B-C and S7C**). We FACS purified GFP<sup>+</sup> (EC) and GFP<sup>-</sup> (non-EC)  
 226 from injured and uninjured tailfins, extracted RNA and performed real time PCR. For *kdm1a*,  
 227 we found no difference in gene expression between groups (**Fig. S8A**). We also analysed the  
 228 expression of CoREST (*rcor1* and *hdac1*) and NuRD (*rbbp4* and *chd4*) factors and did not  
 229 detect significant differences between groups (**Fig. S8B-E**). In response to stress or injury,  
 230 EC upregulate *inos*, with consequent NO production<sup>25</sup>. We found an upregulation of *nos2b*  
 231 compared to *nos2a* and *nos1* genes in the EC of injured tailfins compared to uninjured (**Fig.**  
 232 **S8F-H**). Therefore, we investigated if S-nitrosylation, more specifically of Kdm1a, occurs in  
 233 EC during tailfin regeneration. We observed an overall increase in S-nitrosylated proteins in  
 234 the EC of regenerating tissue compared to EC from uninjured tissue (**Fig. 5D**), with no  
 235 change in S-nitrosylated proteins in GFP<sup>-</sup> cells from injured versus uninjured tailfins. Then,  
 236 we specifically analysed S-nitrosylated Kdm1a in EC cells from injured and uninjured tailfins  
 237 at 5 dpa, including an injured group treated with the nitric oxide (NO) scavenger PTIO.  
 238 While the expression of total *kdm1a* did not change in the three groups, S-nitrosylated  
 239 Kdm1a was detectable in EC from regenerating tailfin only (**Fig. 5E**), while it was abolished

in the group treated with PTIO. The increased S-nitrosylation of Kdm1a in EC of the regenerating tailfin was associated with a significant increase in vessel density compared to the other two groups (**Fig. 5F**).

S-nitrosylation of Kdm1a is associated with increased H3K4me2 marks for endothelial genes during tailfin regeneration.

As shown in **Fig. 4D-E**, H3K4me2, a target of Kdm1a, is increased during regeneration associated with the reduced demethylase activity of the S-nitrosylated form of Kdm1a. Methylated H3K4 is associated with active gene transcription. Accordingly, we reasoned that an increased occupancy of H3K4me2 would result in increased levels of proangiogenic factors. To address this hypothesis, and investigate whether the increased vessel density observed during regeneration was correlated to S-nitrosylation of Kdm1a, we performed Chromatin Immunoprecipitation using a ChIP-grade antibody specific for H3K4me2, followed by PCR for 10 well-known proangiogenic factors, including *kdr*, *vegfaa*, *fgf2*, *angpt2*, *tek*, *tie1*, *cdh5*, *cd31*, *mmp2* and *tbx20*. ChIP-PCR showed that the occupancy of H3K4me2 on *vegfaa* and *tek* in endothelial cells from injured tailfins was significantly higher compared to injured tailfins treated with PTIO and higher than uninjured tailfins (**Fig. 5G**). Furthermore, real time PCR showed that expression of 7 out of 10 proangiogenic factors, including *vegfaa* and *tek*, were significantly increased during regeneration (**Fig. 5H**), whereas they were unchanged following treatment with PTIO, again suggesting that nitric oxide was driving or facilitating angiogenesis via a mechanism involving S-nitrosylation. Overall, these findings show that endothelial cells of the regenerating tissue are a key cell target for protein S-nitrosylation after injury and that Kdm1a is specifically S-nitrosylated in these cells. S-nitrosylation of Kdm1a reduces Kdm1a demethylase activity and, as a

consequence, more H3K4me2 will accumulate in the genome. In particular, we found increased occupancy of H3K4me2 on *vegfaa* and *tek*.

This does not exclude, however, that other S-nitrosylated proteins among those arising from the mass-spec dataset could be implicated in endothelial cells and vascular regrowth as well as in other cell types.

Next, to investigate the effects of the absence of the Kdm1a C334 S-nitrosylation site on tailfin regeneration we synthesized, by site-directed mutagenesis, a mutant variant of Kdm1a mRNA where the cysteine 334 was substituted with alanine (C334A), that cannot be S-nitrosylated (**Fig. 6A**). These experiments assessed the ability of this mutated Kdm1a mRNA to rescue the morpholino (Mo) phenotype compared to wild-type mRNA. However, contrary to the MO oligo that is a stable molecule, not degraded by the nucleases, the mRNA is prone to degradation by nucleases, well before reaching the tailfin, *i.e.* the tissue under study. Therefore, we decided to perform these studies in the zebrafish embryos where the cytoplasmic bridges connecting the early embryonic cells allow rapid diffusion of mRNA into the cells, resulting in fast and ubiquitous delivery<sup>26</sup>.

Before the morpholino (Mo) studies, real time PCR analysis confirmed *kdm1a* expression during development, with a slight reduction from 24 to 120 hpf (**Fig. S9A**). This finding was also useful to optimise the dose of Mo to inject. Mo experiments were conducted according to the guidelines<sup>26,27</sup> using several controls to assess morpholino specificity for *kdm1a*. First, we injected *kdm1a*-targeted Mo or the mismatch (control), using an optimized dose of 0.8 ng per egg. The effective *kdm1a* KD was confirmed by western blotting (**Fig. 6B**). The survival rate of *kdm1a* KD embryos at 120 hpf was approximately 80 % compared to 90 % in controls (**Fig. S9C**). *Kdm1a* KD embryos did not show gross abnormalities compared to control (**Fig. S9D**), however we found reduced blood flow velocity (**Fig. S9E**) and reduced expression of

*gatal* (**Fig. S9F**), a red blood cell marker, consistent with a previous report<sup>28</sup>. To confirm these effects and to exclude sequence-specific off-target effects we used a second Mo with non-overlapping sequence to compare phenotypes with the first Mo. Indeed, both Mo injections induced *kdm1a* knockdown and produced a comparable phenotype. To further confirm the specificity, we co-injected half-doses of each Mo, such that the phenotype was only just apparent with each Mo alone, but with clear additive effects on phenotype when co-injected. Specifically, the two *kdm1a* -targeted antisense Mo were co-injected each at 1/2 dose (0.4 ng per egg). While the phenotype was apparently unaffected with 1/2 dose of each oligo injected alone, the co-injection of half doses together produced phenotypic effects similar to those produced by a single oligo at full dose (0.8 ng per egg) (**Figs. 6B and S9**). These additive effects of low doses of two antisense oligos strongly supports a *kdm1a* - specific effect. We did not find an increase in expression of *tp53*, well-known off-target effect, at the dose of Mo used in this study, therefore we did not co-inject *tp53*-Mo for *tp53* gene silencing (**Fig. S9B**). We observed that the regeneration of the tailfin was significantly reduced in *kdm1a* KD embryos compared to control (**Fig. 6C-D**), with a phenotype penetrance of >70%. The phenotype of *kdm1a* morphants was rescued by co-injecting *kdm1a* mRNA indicating that the observed effects were specific for *kdm1a*. This also clearly shows a key role of *kdm1a* in tailfin regeneration. However, *kdm1a* mRNA C334A did not rescue tailfin regeneration (**Fig. 6C-D and Fig. S9**). This further confirmed the crucial role of S-nitrosylation of this specific cysteine residue on Kdm1a in modulating tailfin regeneration.

## Discussion

Following stress or injury, *inos* is activated within local somatic cells<sup>29</sup>, leading to protein S-nitrosylation, the covalent attachment of a NO group to the thiol side chain of the cysteine.

This mechanism has emerged as a dynamic, post-translational regulatory mechanism for many classes of proteins<sup>30</sup>. We found increased expression of *inos* in the nuclei of the regenerating tailfin of the zebrafish, and over 500 nuclear proteins that became S-nitrosylated during regeneration. Of these, we identified a key role for Kdm1a. We demonstrated a strong link between Kdm1a S-nitrosylation and its role in histone demethylation and ultimately in regeneration of the zebrafish tailfin. Endothelial cells, where *inos* expression increases after stress or injury, seems to be the main cell target where S-nitrosylation, including of Kdm1a, mostly occurs. The essential role of Kdm1a in hematopoiesis has been shown in vitro<sup>31,32</sup> and in vivo<sup>33,34</sup>. As such, understanding the molecular mechanisms underpinning the action of Kdm1a are currently being investigated to find specific inhibitors that could be harnessed as a therapeutic strategy in cancer<sup>35</sup>. According to our bioinformatic analysis of single cell-RNAseq, *kdm1a* was not detected in hematopoietic cells of the zebrafish regenerating tailfin. Nonetheless, given the role of the Kdm1a in hematopoiesis we cannot exclude the possibility that impaired fin regeneration in *kdm1a* knockdown zebrafish could, at least partially, derive from systemic effects associated with altered immune cell function.

In our study, the S-nitrosylated form of Kdm1a has a reduced demethylase activity on H3K4 that results in a corresponding increase in H3K4me2 that, by turn, promotes the expression of proangiogenic genes. This is the first study to show the importance of the S-nitrosylation of nuclear proteins in tissue regeneration and potentially opens up new therapeutic avenues (**Fig. 6E**).

While many interesting candidate S-nitrosylated nuclear proteins were identified (**Dataset S1 and Table S1**), we focused on Kdm1a. However, we predict that it is likely that Kdm1a is not the only rate limiting factor. It is likely that modulating the expression and S-nitrosylation of other candidates could result in similar effects on regeneration, possibly by acting via the same cells and pathways identified here but possibly on other cell types and pathways. This

would depend on the effects that S-nitrosylation has on molecular networking of each protein candidate. For example, S-nitrosylation of Hexim1 (hexamethylene bisacetamide inducible protein 1) could affect its binding in the P-TEFB complex<sup>36</sup> and potentially inhibits the Cdk9 kinase activity and the transcription of genes specific to regeneration. Aberrant levels of protein S-nitrosylation have been implicated in a number of diseases, including heart disease, diabetes, cancer, neurological disorders, chronic degenerative diseases, and inflammatory disorders (reviewed in<sup>37</sup>), regeneration. Furthermore, very little is known about two important aspects of this process, firstly the extent and the dynamics of S-nitrosylation of *nuclear* proteins in human disease and secondly the role of S-nitrosylation of nuclear proteins during tissue repair and regeneration<sup>38</sup>. Our paper is the first to examine the mechanisms and potential role of S-nitrosylation of nuclear proteins during tissue regeneration in vivo and provides several important observations that implicate S-nitrosylation of nuclear proteins in regeneration.

## **Methods**

### Ethical approval

This work complied with all relevant ethical regulations for animal testing and research. Animals were housed and all experiments were carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee at the Houston Methodist Research Institute, and with the United Kingdom Animals (Scientific Procedures) Act 1986 at the Queens Medical Research Institute research facilities.

### Zebrafish aquaculture and husbandry

Adult zebrafish – wild-type *Wik* and *Tg(nfkb:EGFP)<sup>nc1</sup>* strains – were maintained according to standard procedures. Fish were kept at 28 °C under a 14/10 h light/dark cycle and fed with



dry meal (Gemma Micro, Westbrook, ME) twice per day. Embryos were obtained by natural mating and kept in E3 embryo medium at 28.5°C. Surgical procedures were performed under anaesthesia with Tricaine (also named MS-222, Sigma-Aldrich, St Louis, MO, cat. E10521) 0.02 mg/ml on embryos and 0.05 mg/mL in adult zebrafish.

#### Zebrafish tailfin amputation and regeneration

Caudal tailfin amputation surgeries were performed as previously described<sup>39</sup>. Briefly, fish were anaesthetized and amputations were made by using a sterile razor blade, removing half of the tailfin. At day 3, 5 and 10 post-amputation (dpa) (uninjured tailfin was used as control) the regrown tissue was carefully resected and immediately processed for nuclear protein extraction. Total regeneration was measured as previously described<sup>40</sup>. Briefly, fin images were collected before amputation and time points after amputation. The new tissue area (in pixels) of the caudal fin from the new distal fin edge to the amputation plane was quantified in each fish using Image J software. The percentage of regeneration for each fin at each time point was defined as percentage of regeneration = 100 x (regenerated tissue area/original fin area amputated). The collection of the distal (blastema) and proximal (regenerating) regions of the tailfin tissue for PCR analysis was performed under a fluorescence stereomicroscope Leica M205. The *Tg(fli1:EGFP)* zebrafish was used and the distal ends of the newly forming (GFP<sup>+</sup>) vessel branches was used as boundary to separate by dissection the two regions. Then, we placed the tissues in an Eppendorf tube and immediately extracted RNA that was used for PCR analysis.

#### Blood vessel density

At 5 dpa, the adult *Tg(fli1:EGFP)* zebrafish were anesthetized. Then, fish were transferred on a wet sponge, previously soaked in tank water, to keep the zebrafish skin moist during imaging. Images of the caudal fin including the regenerated tissue were captured under a fluorescence stereomicroscope Leica M205 equipped with a camera. The images were collected with a Leica LAS X software and analysed by Image J software. The second and third rays from the dorsal edge of the fin were used for measurement of vessel area and reported as mm<sup>2</sup>.

## Protein Extraction

*Extraction of nuclear proteins* - Nuclear proteins were extracted from the caudal fin tissue using the NE-PER Extraction Reagents kit (Thermo Fisher Scientific, San Jose, CA, cat. 78833) according to the manufacturer's instructions, supplemented with protease inhibitor cocktail. In brief, regenerating tailfin tissue was resected, cut into small pieces and placed in a microcentrifuge tube. Then, tissue was washed three times with chilled PBS, centrifuged at 500g for 1 min and supernatant discarded. Using a motor-driven pestle (Sigma-Aldrich, St Louis, MO, cat. Z359971), tissue was homogenized in solution CER I, that breaks plasma membrane but not nuclei, added with protease/phosphatase inhibitors cocktail (1:100, Thermo Fisher Scientific, San Jose, CA, cat. 78442). The tube was vigorously vortexed for 30 s and put on ice for 10 min. Then, chilled CER II was added to the tube, vortexed for 10 s and incubated on ice for 1 min. The tube was centrifuged for 5 min at 16,000g and the supernatant, containing cytoplasmic proteins, was transferred to a clean pre-chilled tube and stored at -80 °C. The pellet, containing nuclei, was resuspended in chilled NER solution, vortexed on the highest setting for 15 s. The sample was placed on ice for 45 min, vortexed

for 20 s every 10 min. Then, the tube was centrifuged at 16,000g for 10 min and the supernatant, containing nuclear extract, immediately transferred to a clean pre-chilled tube.

*Extraction of total proteins* - Zebrafish embryos were euthanised with an overdose of tricaine, washed three times in PBS and homogenized with a motor-driven pestle (Sigma-Aldrich, St Louis, MO, cat. Z359971) in 100 mL RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), supplemented with protease/phosphatase inhibitors. The lysate was kept on ice for 40 min. Then, the tube was centrifuged at 3000g for 5 min and the supernatant transferred to a clean pre-chilled tube.

In both nuclear and total proteins extraction, bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, San Jose, CA, cat. 23225) was used to measure protein concentration.

#### Labelling of protein S-nitrosothiols

Labelling of S-nitrosothiols in nuclear proteins was achieved using Iodoacetyl Tandem Mass Tag (iodoTMT) kit (Thermo Fisher Scientific, San Jose, CA, cat. 90103). First, nuclear protein extracts were acetone-precipitated at -20°C for 2 h, then centrifuged at 15000g for 10 min and the pellet solubilized in 500 mL HENS buffer (Thermo Fisher Scientific, San Jose, CA, cat. 90106) at a protein concentration of 1 mg/ml. Equal amounts of nuclear protein from each sample were iodoTMT-labelled. To generate a positive control sample, an aliquot of protein from control was added with 200µM S-nitrosoglutathione (GSNO, Sigma-Aldrich, St Louis, MO, cat. N4148) for 30 min at room temperature (RT). Experimental samples were incubated for 30 min at RT after adding MMTS (10µL of 1 M) to block free cysteine thiols. Then, proteins were precipitated with pre-chilled acetone (1 ml per sample) at -20°C for 2 h to remove MMTS. Samples were centrifuged at 10,000g for 10 min at 4°C, the pellet resuspended in 500 ml of HENS buffer and to each was added 5 ml of iodoTMT reagent,

previously dissolved in liquid chromatography/mass spectrometry (LC/MS)-grade methanol, and 10 $\mu$ L of 1M sodium ascorbate (Sigma-Aldrich, St Louis, MO, cat. A4034). As a negative control, 10 $\mu$ L of ultrapure water instead of sodium ascorbate was added in a protein sample. All samples were incubated for 1 h at 37°C, protected from light. The reaction was quenched by adding 20  $\mu$ L of 0.5M DTT and incubated for 15 min at 37°C, protected from light. All experimental samples labelled with iodoTMT sixplex were combined, added with six volumes of pre-chilled acetone and incubated at -20°C overnight. The sample was centrifuged at 10,000g for 10 min at 4°C and the pellet dissolved in 3 ml HENS buffer. Then, 100 $\mu$ L of 0.5 M iodoacetamide were added and the sample incubated at 37°C for 1 h protected from light. Sample was precipitated with pre-chilled acetone and the pellet allowed to dry for 10 min.

#### Protein digestion for mass-spec analysis

The pellet was dissolved in 50mM ammonium bicarbonate (Sigma-Aldrich, St Louis, MO, cat. 09830) and digested using trypsin enzyme (GenDepot, cat. T9600) at 37°C overnight. The peptide mixture was acidified using 10% formic acid and dried using a vacuum concentrator (Thermo Fisher Scientific, San Jose, CA, cat. SPD120).

#### Enrichment of iodoTMT-labeled S-nitrosopeptides

The anti-TMT Antibody Resin (Thermo Fisher Scientific, San Jose, CA, cat. 90076) was washed three times with Tris Buffered Saline (TBS) (Thermo Fisher Scientific, San Jose, CA, cat. 28358). Previous labeled and lyophilized peptides were resuspended in TBS (a small portion of unfractionated sample was stored for direct analysis of the non-enriched samples). Then, peptides were added to the anti-TMT resin (100 $\mu$ L of settled resin for every 1 mg of

iodoTMT Reagent-labeled peptides) and incubated at RT for 4 h. Finally, the resin was washed three times (5 min per wash) with TBS and then three times with water. The sample was eluted with TMT Elution Buffer (Thermo Fisher Scientific, San Jose, CA, cat. 90104). The eluate was frozen and lyophilized, using a vacuum concentrator and then the sample resuspended in a solution of 5% methanol/0.1% formic acid. Then, 1-5 $\mu$ L of sample were injected directly onto an LC-MS/MS system.

#### LC/MS-MS

The mass spectrometry analysis of S-nitrosopeptides was carried out on a nano-LC 1200 system (Thermo Fisher Scientific, San Jose, CA) coupled to Orbitrap Fusion™ Lumos ETD (Thermo Fisher Scientific, San Jose, CA) mass spectrometer. The peptides were loaded onto a Reprosil-Pur Basic C18 (1.9  $\mu$ m, Dr. Maisch GmbH, Germany) pre-column of 2 cm X 100  $\mu$ m and in-lined an in-housed 5 cm x 150  $\mu$ m analytical column packed with Reprosil-Pur Basic C18 beads. The peptides were separated using a 75 min discontinuous gradient of 5-28% acetonitrile/0.1% formic acid at a flow rate of 750nl/min. The eluted peptides were directly electro-sprayed into the mass spectrometer. The instrument used the multi-notch MS3-based TMT method. The full MS scan was performed in Orbitrap in the range of 375-1500m/z at 120000 resolution followed by ion trap CID-MS2 fragmentation at precursor isolation width of 0.7 m/z, AGC of 1X104, maximum ion accumulation time of 50ms. The top ten fragment ions from MS2 was selected for HCD-MS3 with isolation width of 2 m/z, AGC 5X104, collision energy 65%, maximum injection time of 54ms. The RAW file from mass spectrometer was processed with Proteome Discoverer 2.1 (Thermo Fisher Scientific, San Jose, CA) using Mascot 2.4 (Matrix Science) with percolator against Zebrafish Uniprot database. The precursor ion tolerance and product ion tolerance were set to 20 ppm and

0.5Da respectively. Variable modifications of oxidation on Methionine (+15.995Da) and iodoTMT tag (+329.2266Da) on cysteine residues was used. The general quantification in consensus workflow used unique and razor peptides with top 3 peptides for area calculation, while reporter quantification used co-isolation threshold of 50 and average reporter S/N threshold of 10. The assigned peptides and PSMs were filtered at 1% FDR.

#### Bioinformatic analysis

The computational detection strategy identifies peptides exhibiting iodoTMT tag modifications. Proteome Discoverer software (Thermo Fisher Scientific, San Jose, CA) was used to search MS/MS spectra against the Zebrafish UniProt database (Danio rerio; UP000000437) using Mascot 2.3 search engine. The iodoTMTsixplex quantification method within Proteome Discoverer software was used to calculate the reporter ratios with a mass tolerance  $\pm 10$  ppm. Search algorithms, including MS-Fragger was also used in the analysis. Hierarchical clustering of S-nitrosylated protein expression heatmap was conducted using MEV based on Pearson correlation distance metric and the average linkage method. Ingenuity pathway analysis (IPA, Ingenuity systems Qiagen, Redwood City, CA) was used to assess Gene Ontology (GO) and IPA analysis to explore the function of differential S-nitrosylated proteins. Enrichment Q values of S-nitrosylated protein pathways were defined based on EdgeR FDR cutoff  $1e-5$ . The GO category was classified by Fisher's exact test, and the p-value was corrected by the false discovery rate (FDR) calculation.

The scRNA-seq of the regenerative caudal fin was analysed following the methods described in the manuscript<sup>23</sup>. The matrix count from cell ranger were obtained on GEO database accession GSE137971. Downstream analysis was performed on R using the package Seurat. Clustering analysis was performed on the integrated dataset and we found 6 clusters. These

clusters were annotated as Superficial/intermediate/basal epithelial, mucosal-like, hematopoietic and mesenchymal based on markers described in Hou et al.<sup>23</sup>. Differential gene expression between the different time points compared to pre-injury was done using the function FindMarkers from Seurat (based on Wilcoxon test followed by Bonferroni correction). Violin Plot of *kdm1a* expression was obtained using the Vln Plot function of Seurat.

#### Chromatin immunoprecipitation (ChIP)-PCR assay

ChIP assay was performed following the manufacturer's instructions (Cell Signalling Technology, Beverly, MA). Briefly, 15 tailfins of adult zebrafish per group were disaggregated in single cells as described above. DNA and protein were crosslinked by 1% formaldehyde. Chromatin was isolated and digested with Micrococcal Nuclease. Then, the DNA-protein complex was precipitated with control IgG or antibody against H3K4me2 (rabbit polyclonal, ChIP grade) overnight at 4°C and protein A/G conjugated magnetic beads for 1 hr. Cross-links were reversed. The extracted DNA was used as template for PCR amplification of the targeted promoter region. The extracted DNA from unprecipitated DNA-protein complex was used as input. The promoter regions of 10 genes known to be involved in neoangiogenesis (*kdr*, *vegfaa*, *cdh5*, *tek*, *tie2*, *tbx20*, *fgf2*, *angpt2*, *mmp2*, *cd31*) were identified in ENSEMBL. The gene sequence up to 600 bp upstream of the TSS was validated this sequence on genome.ucsc.edu to confirm it was upstream of our gene of interest. We also looked for the presence of CpG islands and TATA box. Hence, we designed four couples of primers using Primer Blast for each gene that matched in this region and around the TSS, and that could generate amplicons which size was no more than 120-130 bp to allow both primers to find their target on one fragment of ChIP DNA, if present. In-silico PCR (UCSC) was used

to confirm that primers matched our region of interest and the amplicon size, and then primers were further validated by PCR using genomic DNA.

#### Quantification of Kdm1a demethylase activity

Kdm1 Activity Colorimetric Kit (Abcam, Cambridge, UK, cat. ab113459) was used to quantify Kdm1 activity. Nuclear proteins were extracted from the regenerating caudal fin as shown above and an input of 10µg per sample was used for the enzymatic analysis. The experiment was run in triplicate. A standard curve was prepared with Kdm1a assay buffer and assay standard solution, containing demethylated histone H3K4, diluted at concentration between 0.2 and 5 ng/µl. Sample wells were added with Kdm1a assay buffer, Kdm1a substrate (containing di-methylated histone H3K4) and 10µg of nuclear extract. No nuclear extract was added in blank wells. The strip-well microplate was covered with adhesive film to avoid evaporation, and incubate at 37°C for 2 h. At this stage, active Kdm1a binds to the substrate and removes methyl groups from the substrate. Then, the reaction solution was removed and each well washed three times with wash buffer. Capture antibody, that recognizes Kdm1a-demethylated products, was added to each well, the strip-well was covered with aluminum foil to protect from light and incubated at RT for 60 min. Antibody solution was removed and each well washed three times with wash buffer. Then, detection antibody was added to each well, covered again with aluminum foil and incubated at RT for 30 min. Detection antibody solution was removed and each well was washed four times with wash buffer. Developer solution was added and the microplate incubated at RT for 10 min protected from light. In presence of methylated DNA, the solution will turn blue. At this point, stop solution was added to each well to quench the enzymatic reaction. Absorbance was read on a microplate reader Infinite M1000 (Tecan, Männedorf, Switzerland) at a



wavelength at 450 nm with an optional reference wavelength of 655 nm. The activity of *Kdm1a* enzyme is proportional to the optical density (OD) intensity measured. Accordingly, *Kdm1a* activity was calculated using the following formula:

$$\text{Kdm1a activity (OD/min/mg)} = \frac{\text{Sample OD} - \text{Blank OD}}{\text{Protein Amount } (\mu\text{g}) \times \text{min}}.$$

#### *Kdm1a* suppression

The knockdown (KD) of *kdm1a* gene (NM\_001242995.1) in zebrafish was achieved by injection of antisense morpholino (Mo) (Gene Tools, Philomath, Oregon) oligo targeting the mRNA AUG translational start site (sequence 5'-TTGGACAACATCACAGATGACAGAG-3'). A 5-base pair mismatch Mo (sequence 5'-TTGcAgAACATgACAcATcACAGAG-3') was used as control to detect possible off-target effects. A second antisense oligo targeting i3e4 splice junction of *kdm1a*, sequence 5'-CTACACCTGAGAAACCCAACATTTC-3' was used to corroborate data obtained with MOs that block translation.

Using a standard microinjector (IM300 Microinjector; Narishige, Tokyo, Japan), an optimized dose of 0.4 ng (0.5nL bolus) of morpholino placed in a pulled glass capillary was injected in each embryo at 1–2 cell stage, just beneath the blastoderm.

For KD of *kdm1a* in adult zebrafish, a vivo-Mo version was used, where the standard Mo is bound to a synthetic scaffold containing guanidinium groups as a delivery moiety in adult tissues. An antisense vivo-Mo that targets human b-globin intron mutation 5'-

CCTCTTACCTCATTACAATTTATA-3' was used as negative control (Gene Tools LLC, Philomath, Oregon). Adult zebrafish were anesthetized in tricaine 0.05 mg/mL, and 2  $\mu$ L of 0.1 mmol/L vivo-Mo solution, previously loaded in a glass capillary, was injected into the retro-orbital vein, as previously described<sup>41</sup>, on days 10, 8, 6, 4, 2 and 0 before tailfin amputation.

575 *kdm1a* construct

576 A construct with *kdm1a* gene of *Danio rerio* (NM\_001242995.1) was prepared to generate  
577 *kdm1a* modified (m) mRNA and was assembled from synthetic oligonucleotides.  
578 Modifications in the triplet code (n=7 silent mutations) were inserted in the sequence  
579 corresponding to the mRNA AUG translational start site (*i.e.* Mo binding site) to prevent Mo  
580 recognition in rescue experiments. The fragment was inserted in the pMA (GeneArt,  
581 Invitrogen, Carlsbad, CA) cloning vector and cloned in transformed *Escherichia coli* bacteria  
582 (strain K12/DH10B, Invitrogen, Carlsbad, CA) and then purified. The final construct was  
583 verified by sequencing and the sequence identity within the insertion site was 100%.

584

#### 585 Site-Directed Mutagenesis

586 Site-directed mutagenesis of *Kdm1a* was carried out with Q5 Site-Directed Mutagenesis kit  
587 (New England Biolabs, Ipswich, MA, cat. E0554) according to manufacturers' instructions.  
588 Standard primers for *kdm1a* were used for exponential amplification of the plasmid DNA (F  
589 5'-GACAGCCAGTCGAGGAGAAC-3' and R 5'-TGCGACGTACGAGTATGAGC-3'),  
590 and mutagenic primers to create substitution of Cys334-to-Ala (C334A) in the plasmid were  
591 designed with the software NEBase Changer (F 5'-AAACAGAAGGCTCCCCTCTATGA  
592 GGC-3' and R 5'-ATCTTAGCCAGCTCCATATTG-3').

593

#### 594 In vitro transcription of *kdm1a*

595 Wild type and mutated *kdm1a* mRNA (C334A), with 7-methyl guanosine cap structure at the  
596 5' end and poly(A) tail at the 3' end, was transcribed from the constructs using HiScribe T7  
597 ARCA mRNA Kit (New England Biolabs, Ipswich, MA, cat. E2060) following  
598 manufacturers' instructions.

599 Rescue of *kdm1a* knockdown by Kdm1a mRNA

600 To determine whether the effects of the *kdm1a* KD on zebrafish embryos phenotype and  
601 tailfin regeneration were specifically due to loss of *kdm1a*, we co-injected *kdm1a* -Mo with  
602 *kdm1a* mRNA wild-type as a rescue. A bolus of 1 nl of solution containing 0.5 ng of *kdm1a*-  
603 Mo a and 1 ng of Kdm1a RNA wild-type was injected into each egg.

604

605 Rescue of *kdm1a* knockdown by kdm1a mRNA C334A

606 Co-injection of *kdm1a* -Mo and *kdm1a* mRNA C334A was performed to assess whether the  
607 absence of the S-nitrosylated cysteine affected the ability of the mRNA to rescue phenotype  
608 and tailfin regeneration associated with *kdm1a* KD. A bolus of 1 nl of solution containing 0.5  
609 ng of *kdm1a* -Mo and 1 ng of *kdm1a* mRNA C334A was injected into each egg.

610

611 Pharmacological modulation of S-nitrosylation

612 Adult zebrafish were anesthetized in tricaine 0.05 mg/mL. A solution of 2 µL of iNos  
613 inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich, St Louis, MO, cat.  
614 N5751) 10 or 50 mM diluted in sterile PBS (from stock solution of 250 mM), or of nitric  
615 oxide (NO) scavenger Phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide (PTIO, Sigma-  
616 Aldrich, St Louis, MO, cat. P5084) 3 or 10 mM diluted in sterile PBS (from stock solution of  
617 100 mM), or of NO donor S-Nitroso-N-acetyl-DL-penicillamine (SNAP, Sigma-Aldrich, St  
618 Louis, MO, cat. N3398) 10 or 30 mM diluted in DMSO (from stock solution of 100 mM), or  
619 PBS as control, was loaded on a glass capillary, prepared in advance with a micropipette  
620 puller (Narishige, Inc., PC-10) and connected to a microinjector (IM300 Microinjector;

Narishige, Tokyo, Japan) and was injected into the retro-orbital vein as previously described<sup>41</sup> on days 6, 4, 2 and 0 before tailfin amputation.

The concentration of the Nos inhibitors, L-NAME and 1400W, were adopted after a pilot study with doses up to 250 mM. The survival was recorded and fish were monitored for any physical or behavioral abnormalities at a range of doses. For both compounds at a dose of 250 nM, survival after 4 injections was approximately 40%, and lethargy and reduced swim were observed. At 150 mM the survival increased to 65% with no evident abnormal behaviour; whereas at 50mM, the dose e adopted for the study, the survival was 85% and no evident defects were observed (**Fig. S2B**).

#### Optimisation of the injection procedure

In our pilot studies, injection of physiological solutions every other day and alternating the eye at each injection reduced fish mortality. In this way, the effect of a drug on fish survival/mortality and phenotype can be better evaluated. Therefore, all the solutions of drugs were injected in the retro-orbital veins every other day and alternating the eyes, so that each eye was injected only twice at a distance of four days.

#### Defining the zebrafish phenotype

Whole embryo phenotype following Mo and mRNA treatments were described on the basis of the following morphologic features observed under bright-field microscopy: reduced body length, curved body, reduced swimming, chorionated larvae at 4 dpf, oedema. The phenotype was assessed using a simple six points scoring system, according to the severity of that feature and where one point was normal. At least four different clutches of larvae were assessed under each of the treatment groups. Data were reported graphically as divided in two

groups: normal, *i.e.* embryos not showing any abnormal features, and abnormal, *i.e.* embryos showing one or more of the features described above.

#### Cardinal vein blood velocity

Blood velocity was estimated in the posterior cardinal vein<sup>42</sup> by assessing frame by frame motion of single blood cells determined from video images captured in the zebrafish tail at the level of the cloaca. Four erythrocytes per fish (at least 5 embryos per group) over 10 frames at video frame-rate of 30 frames per second (fps) were analyzed using ImageJ to determine mean blood cell velocity ( $\mu\text{m}\cdot\text{s}^{-1}$ ).

#### Kaplan-Meier analysis of survival

Kaplan-Meier analysis was used to measure the survival of adult zebrafish or larvae following each defined treatment, using PRISM 7 software.

#### Immunoprecipitation

Immunoprecipitation experiments were performed using the Pierce Classic Magnetic IP/Co-IP Kit (Thermo Fisher Scientific, San Jose, CA, cat. MAN0011737), according to manufacturer's instructions.

#### RNA extraction and quantitative PCR

mRNA was extracted from embryos using column purification (RNeasy Mini Kit, Qiagen, Hilden, Germany, cat. 74104) according to the manufacturer's instructions. Working surfaces

were cleaned with RNase Zap (Thermo Fisher Scientific, San Jose, CA, cat. AM9780) to deactivate environmental RNase. Efficient disruption and homogenization of tissue was done using sterile RNase-free disposable pestles (Thermo Fisher Scientific, San Jose, CA, cat. 12-141-368) mounted on a cordless motor for 30 s and then passing the lysate 5-10 times through the needle (18-21 gauge) mounted on a RNase free syringe. RNA integrity was assessed on basis of 18S and 28S ribosomal RNA (rRNA) bands. mRNA was reverse transcribed into cDNA using qScript cDNA Synthesis Kit (Quanta Bio, Beverly, MA, cat. 95047), Primers (IDT Technologies, Coralville, Iowa) targeting genes of interest (see **table S2**) and SYBR Green PCR kit (Invitrogen, Carlsbad, CA) were used for real-time qPCR, that was performed with the QuantStudio 12 k Flex system (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Gene expression was expressed as relative fold changes using the  $\Delta C_t$  method of analysis and normalized to  $\beta$ -actin.

## Western blotting

Lysates containing 20 $\mu$ g of protein each were added with Laemmli buffer (4X) and deionised water to reach a final volume of 20 $\mu$ l. A sample containing pre-stained protein standard (BioRad, Hertfordshire, UK, cat. 1610375) was used to assess molecular mass of protein bands. Samples were heated at 95°C for 5 min and loaded on a polyacrylamide gel (4-15% gradient) (BioRad, Hertfordshire, UK, cat. 4561083). Electrophoresis was performed for 30 min at a voltage of 100V and then for 60 min at 150V. Gels were transferred on PVDF membranes (Amersham Hybond, Sigma-Aldrich, St Louis, MO, cat. GE10600023) for 2 h at 100V. Membranes were blocked with non-fat milk 5% in PBST (PBS+0.1% Tween) for 1h at RT and probed with primary antibody overnight at 4°C. Antibodies used were: Kdmla rabbit polyclonal (1:200, Thermo Fisher Scientific, San Jose, CA, cat. PA1-41697); anti-iNos

mouse monoclonal (1:200, BD Transduction Laboratories, San Jose, CA, cat. 610432); anti-  
β-tubulin rabbit polyclonal (1:500, Abcam, Cambridge, UK, cat. ab6046), Anti-Histone H3  
nuclear marker, rabbit polyclonal (1:500, Abcam, Cambridge, UK, cat. ab1791); anti-Histone  
H3 (1:500, unmodified Lys4), mouse monoclonal (1:500, Merck Millipore, Massachusetts,  
USA, cat. 05-1341); anti-monomethyl-Histone H3 (Lys4), rabbit polyclonal (1:500, Merck  
Millipore, Massachusetts, USA, cat. 07-436); anti-dimethyl-Histone H3 (Lys4), rabbit  
monoclonal (1:500, Merck Millipore, Massachusetts, USA, cat. 04-790); anti-monomethyl-  
Histone H3 (Lys9), rabbit polyclonal (1:500, Merck Millipore, Massachusetts, USA, cat.  
ABE101); anti-dimethyl-Histone H3 (Lys9) rabbit polyclonal (1:500, Merck Millipore,  
Massachusetts, USA, cat. 07-212); anti-Rcor1 rabbit polyclonal (1:200, Invitrogen, Carlsbad,  
CA, cat. PA5-41564); anti-Hdac1 rabbit polyclonal (1:200, Abcam, Cambridge, UK, cat.  
ab33278); anti-Rbbp4 rabbit polyclonal (1:200, Biorbyt, Cat. orb583248); anti-Chd4 rabbit  
polyclonal (1:200, Biorbyt, Cambridge, UK, cat. orb575051); anti-TMT mouse monoclonal  
(1:200, Thermo Fisher Scientific, San Jose, CA, cat. 90075). Membranes were washed three  
times (5 min per wash) with PBS and incubated with HRP-conjugated goat anti-mouse  
(1:2000, Santa Cruz Biotechnology, Dallas, USA, SC-2005) or anti-rabbit (1:2000, Santa  
Cruz Biotechnology, Dallas, USA, SC-2004) antibodies for 1 h at RT. Then, membranes  
were washed again three times with PBS (5 min per wash). Antigen-antibody complexes  
were detected by incubation for 5 min to the enhanced chemiluminescence solution (ECL,  
Amersham) followed by exposure to a photographic film (BioMax XAR Film Kodak, Sigma-  
Aldrich, St Louis, MO). The film was developed and band density was quantified by  
densitometry using ImageJ. β-tubulin and Histone H3 were used as loading control for  
cytoplasmic and nuclear protein, respectively.

716 Sequence and structural analysis of Kdm1a protein  
 717 Similarities of zebrafish and human Kdm1a proteins were assessed using Protein Blast  
 718 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Kdm1a crystal structure was obtained from the  
 719 Protein Data bank (<https://www.ebi.ac.uk/pdbe/entry/pdb/6nqm>).  
 720  
 721 Enzymatic isolation of endothelial cells from zebrafish tailfin  
 722 Cells were isolated according to <sup>43</sup> with some modifications. In brief, amputated tailfin from  
 723 adult Tg(fli1:EGFP)<sup>y1</sup> zebrafish were placed in chilled PBS, washed with calcium-free  
 724 Ringer solution (116 mM NaCl, 2.6 mM KCl, and 5 mM Hepes, pH 7.0), and replaced with 1  
 725 mL solution of trypsin 0.25% (Gibco) added with 50 µg collagenase P (Roche) and 1 mM  
 726 EDTA. Tissue was disaggregated first using fine scissors and then by pipetting the solution  
 727 with a 200-µL pipette tip every 5 min for about 30 min. Cell suspensions were filtered  
 728 through a 40-µm cell strainer (BD Biosciences) into FACS tubes.  
 729  
 730 Flow cytometry characterization of *fli1*<sup>+</sup> cells from zebrafish.  
 731 Cell samples were run on a BD FACS Aria (BD Biosciences). FSC-H and FSC-A were used  
 732 to select cell singlets; 4',6-diamidino-2-phenylindole (DAPI) to select viable single cells;  
 733 wild-type (Wik) zebrafish were used to set the gate between GFP<sup>-</sup> (*i.e.*, *fli1*<sup>-</sup>) and GFP<sup>+</sup> (*i.e.*,  
 734 *fli1*<sup>+</sup>) cells. At least 10,000 of *fli1*<sup>-</sup> and *fli1*<sup>+</sup> cells (excitation [Ex]: 488 nm; emission [Em]:  
 735 530 nm) were sorted into chilled PBS and 10% fetal bovine serum for further analysis.  
 736 FlowJo 10 (Becton and Dickinson) was used to analyse data.  
 737  
 738 Statistical analysis



Results were expressed as the mean  $\pm$  SEM. Each experiment was performed 3 times (biological replicates). The Shapiro-Wilk test was used to confirm the null hypothesis that the data follow a normal distribution. Statistical comparisons between two groups or multiple groups were then performed, respectively, via Student t-test or ANOVA test using PRISM 7 software followed by Bonferroni post hoc test. Log-rank test and Gehan-Breslow-Wilcoxon test were used for statistical analysis of the Kaplan-Meier curves. A P value  $<0.05$  was considered significant.

#### Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

#### Data availability

TMT-labelled S-nitrosylated protein analysis mass spectrometry data have been deposited to the ProteomeXchange Consortium via the MASSIVE repository (MSV000085055) with the dataset identifier PXD017883[<https://www.ebi.ac.uk/pride/archive/projects/PXD17883>] and are freely available. Furthermore, a full list of the S-nitrosylated proteins derived from the mass-spec is included in this manuscript as Dataset S1. Gene Expression Omnibus (GEO) database, accession GSE137971[<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE137971>] was used for single cell sequencing analysis. All other relevant data supporting the key findings of this study are available within the article supplementary files, and Source Data file.

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## Author information

### Author contributions

G.M. is the senior author of this work and is primarily responsible for the conception, design and experimental investigation, data collection and analysis, resources, and original and revised drafts; S.Y.J., J.M.C., A.J. and H.E.L. contributed to mass spectrometry run and dataset collection; C.C., K.R. and JR contributed to bioinformatic analysis; M.D. and A.H.B contributed resources, advised on experimental design and contributed to manuscript editing and discussions; J.P.C. contributed to conceptualization, resources and editing.

## Ethics declarations

### Competing interests

The authors declare no competing interests.

## Legends

### **Figure 1 – Modulation of Nos and nuclear protein S-nitrosylation during tailfin**

**regeneration in adult zebrafish. A.** Real time PCR for *nos1*, *nos2a* and *nos2b* in tailfin at 3, 5, 10 dpa. **B.** Western blot (WB) analysis of Nos2 in tailfins at 3, 5 and 10 dpa and semi-quantitative analysis of bands. **C.** WB analysis of Nos2 in nucleus and cytoplasm of tailfins at 3, 5 and 10 dpa. Semi-quantitative analysis of bands shows nuclear to cytoplasmic protein ratio. **D.** WB analysis of S-nitrosylated nuclear proteins in the regenerating tailfin. S-nitrosothiols were specifically labelled with TMT. An anti-TMT antibody was used to detect S-nitrosylated proteins. Neg and Pos are respectively the negative (without ascorbic acid) and positive (with S-nitrosoglutathione) controls. **E-F.** WB analysis of S-nitrosylated nuclear proteins in tailfin following treatment with L-NAME 10 or 50 mM, 2-Phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide (PTIO) 3 or 10 mM, S-Nitroso-N-acetyl-DL-penicillamine (SNAP) 10 or 30 mM, or PBS (control). The dot plot shows changes in tailfin regeneration rate, here shown at 7 dpa, following drug treatments compared to control.  $\beta$ -tubulin was used as loading control for total or cytoplasmic proteins. Histone H3 was used as loading control for nuclear proteins. Dpa – days post-amputation. N=3 biological replicates, one way ANOVA test followed by Bonferroni's multiple comparisons test was used to compare the means, p values shown are vs uninjured or control, all other comparisons are not significant. Data are presented as mean values +/- SEM.

### **Figure 2 – Bioinformatic analysis of the S-nitrosylome in zebrafish tailfin regeneration.**

**A.** Workflow for the analysis of the S-nitrosylome. Tailfins of zebrafish (6 months old) were amputated; regrown tissue was collected in uninjured and at 3, 5 and 10 dpa (dash red lines represent the edge of the amputation); nuclear proteins were extracted and labelled with iodoTMT, digested with trypsin, and S-nitroso-peptides enriched through anti-TMT antibody containing resin and followed by LC-MS-MS. **B-C.** Hierarchical clustering heat map and

Venn diagram showing the dynamic changes in the number of S-nitrosylated nuclear proteins during the regeneration compared to uninjured. **D.** Hallmark pathways enrichment by the differentially expressed S-nitrosylated proteins during regeneration compared to uninjured. The significant pathways are displayed along the x-axis. Dpa - day post-amputation. N=2 biological replicates, followed by bioinformatic analysis.

**Figure 3 – Role of S-nitrosylation of Kdm1a in tailfin regeneration in adult zebrafish. A.** MS/MS fragmentation spectrum for the Cys334-containing peptide of Kdm1a. Peptide sequence is shown at the top left of the spectrum, with the annotation of the identified matched amino terminus-containing ions (b ions) in black and the carboxyl terminus-containing ions (y ions) in red. The spectrum confirms the identity of the peptides CPLYEAN and the labeled C as S-nitrosylated cysteine. **B.** Line graph reporting the quantification of Kdm1a S-nitrosylation (normalized by total Kdm1a) and Kdm1a activity during tailfin regeneration. **C.** Western blotting (WB) for S-nitrosylated Kdm1a in uninjured and at 3, 5 and 10 dpa. Samples were previously immunoprecipitated (IP) for Kdm1a. IP with IgG and input were used as controls. **D-E-F.** WB for Kdm1a, CoRest and NuRD complexes components following IP with Kdm1a antibody in tailfin uninjured or injured at 5 dpa. Dpa – days post-amputation. N=3 biological replicates.

**Figure 4 – Effects of *kdm1a* knockdown in adult zebrafish. A.** Western blotting (WB) analysis of Kdm1a control and morpholino KD. Dot plot shows semiquantitative analysis of bands. Two-tailed t-test. **B.** Effects of *Kdm1a* KD on tailfin regeneration. Dashed red line represents the edge of the resection. Scale bar indicates 2 mm. **C.** Line graph showing changes in tailfin regeneration rate following *kdm1a* KD. Two-way ANOVA followed by Bonferroni's multiple comparisons test. **D-E.** WB for H3K4unme (unmethylated), H3K4me1, H3K4me2 and H3K4me3 in control uninjured, injured and injured + *kdm1a* KD at at 5 dpa.



Dot plot shows semi-quantitative analysis of bands. Histone H3 was used as loading control. Dpa - days post-amputation. Two-way ANOVA followed by Bonferroni's multiple comparisons test, p values indicate comparisons of uninjured vs other groups. N=3 biological replicates. Data are presented as mean values +/- SEM.

**Figure 5 – Analysis of S-nitrosylation in endothelial cells during tailfin regeneration. A.**

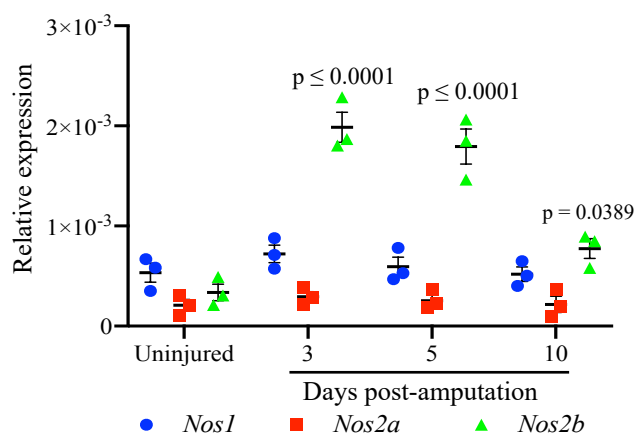
Brightfield and fluorescence images of *Tg(fli1:EGFP)<sup>y1</sup>* zebrafish tailfin at 3 days post-amputation (dpa) showing formation of new vessel branches (GFP signal). Scale bar measures 500  $\mu$ m. **B.** FACS plot of GFP<sup>+</sup> and GFP<sup>-</sup> cells in the tailfin in control and during regeneration were separated by FACS. **C.** Quantification of GFP<sup>+</sup> cells as shown in FACS plots. Two-tailed t-test. **D.** Western blotting (WB) of total S-nitrosylated proteins in zebrafish tailfin endothelial (GFP<sup>+</sup>) cells. **E.** WB of Kdm1a and S-nitrosylated Kdm1a in endothelial (GFP<sup>+</sup>) cells control, injury and injury + PTIO (NO scavenger) 10 mM. Dot plot shows semi-quantitative analysis. p values vs 5 dpa group. **F.** Vessel density analysis in *Tg(fli1:EGFP)<sup>y1</sup>* zebrafish tailfin uninjured, injured and injured + PTIO 10 mM, measured as total length of vessels. **G.** ChIP-PCR analysis in GFP<sup>+</sup> cells isolated from the regenerating tailfin showing H3K4me2-binding complex with *vegfaa* and *tek* promoters. Rabbit IgG were used as a negative control. **H.** Real time PCR analysis for endothelial genes in GFP<sup>+</sup> cells from zebrafish control, injury and injury + PTIO 10 mM. Histone H3 was used as loading control. One-way ANOVA followed by Bonferroni's multiple comparisons test, p values indicate comparisons vs uninjured. N=3 biological replicates. Data are presented as mean values +/- SEM.

**Figure 6. Modulation of Kdm1a S-nitrosylation during tailfin regeneration. A. *Kdm1a***

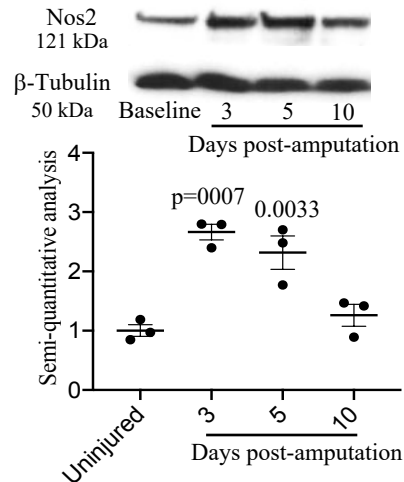
mRNA C334A was generated by site-directed mutagenesis, replacing the aa Cys334 with Ala. B-D. Zebrafish embryos injected with *kdm1a* morpholino (Mo), or co-injected with

960 *kdm1a* Mo with *kdm1a* mRNA C334A or wild type. **B.** Western blotting and semi-  
961 quantitative analysis showed the effective knockdown and rescue of *kdm1a* following the  
962 different treatments.  $\beta$ -tubulin was used as loading control. **C-D.** Images and dot plot of  
963 tailfin regeneration following *kdm1a* modulation (P values vs control). Scale bar measures  
964 100  $\mu$ m. **E.** Working model. Tissue injury promotes the S-nitrosylation of the Cys334 of  
965 Kdm1a. S-nitrosylated Kdm1a detaches from the CoRest complex and loses its demethylase  
966 activity on H3K4. One-way ANOVA followed by Bonferroni's multiple comparisons test, p  
967 values indicate comparisons vs control. N=3 biological replicates. Data are presented as mean  
968 values  $\pm$  SEM.

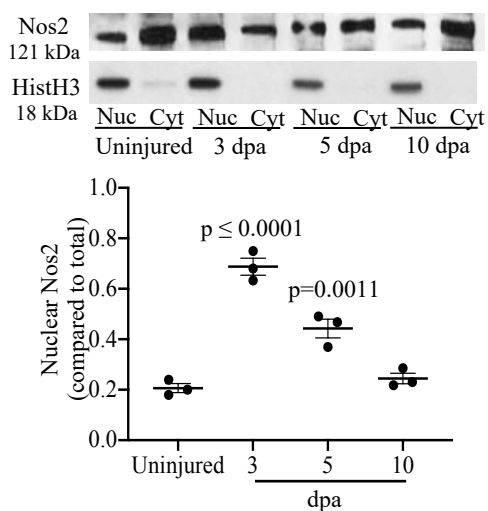
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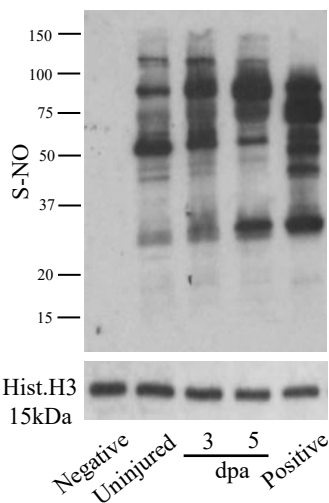
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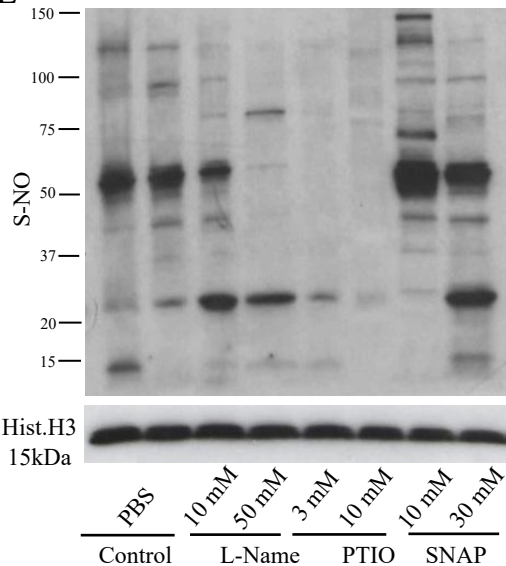
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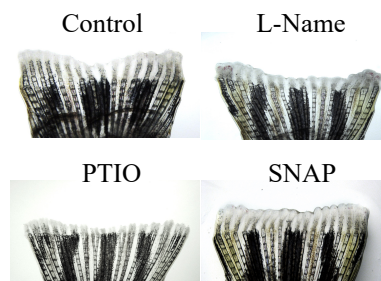
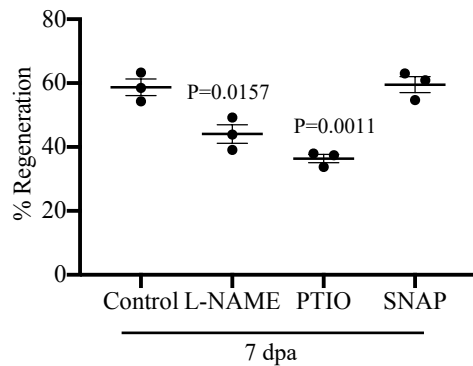
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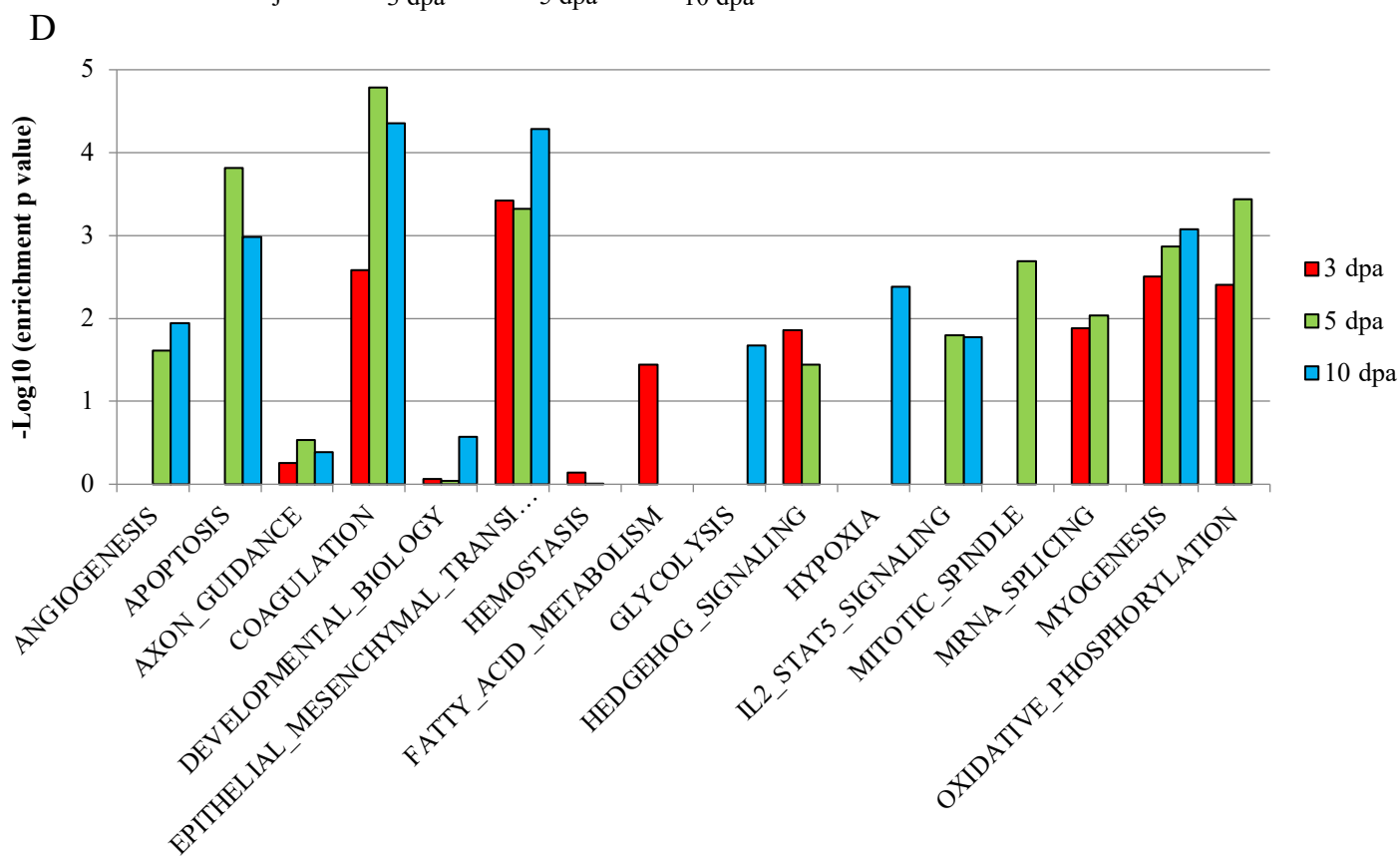
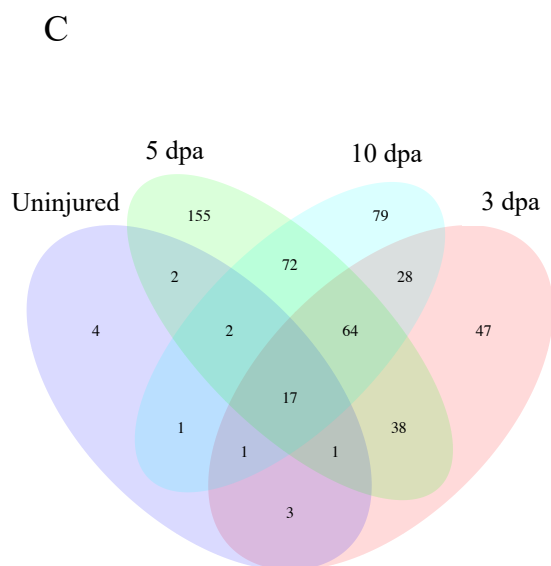
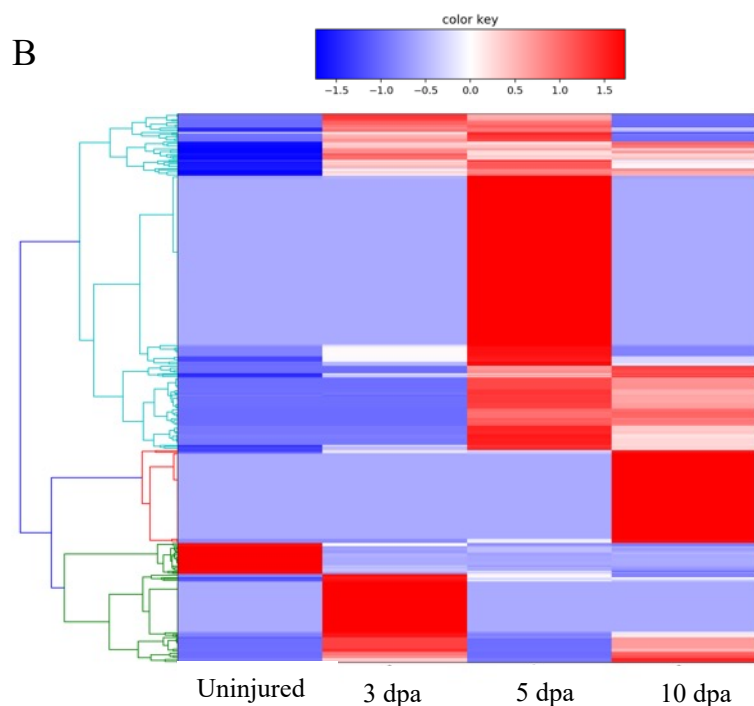
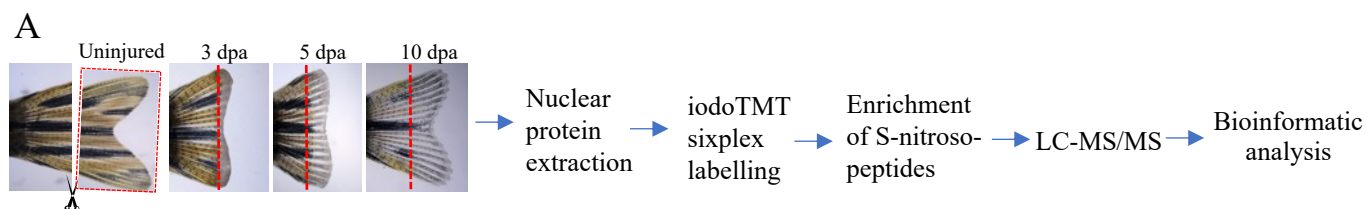


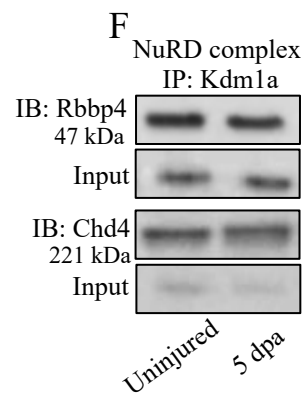
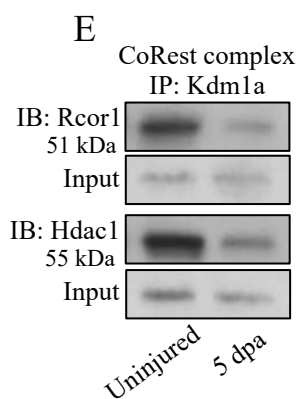
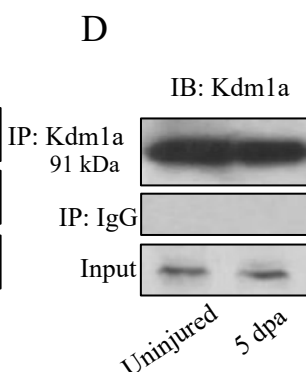
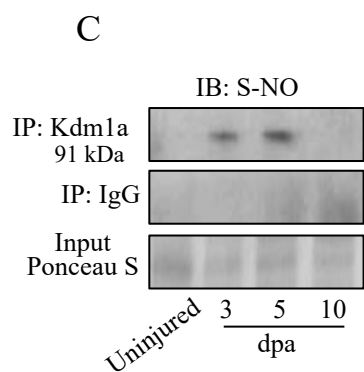
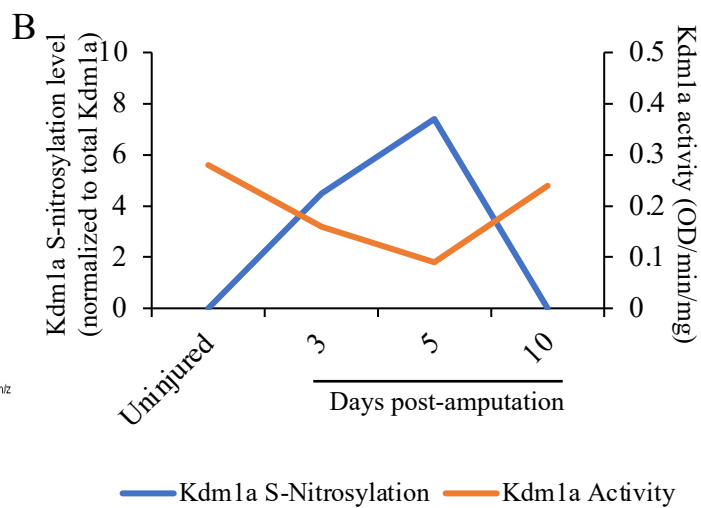
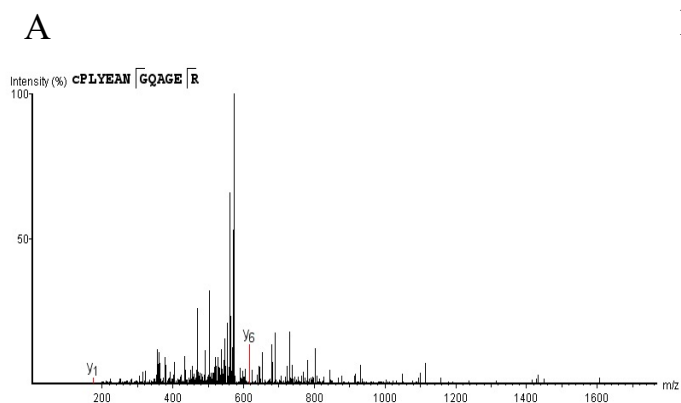
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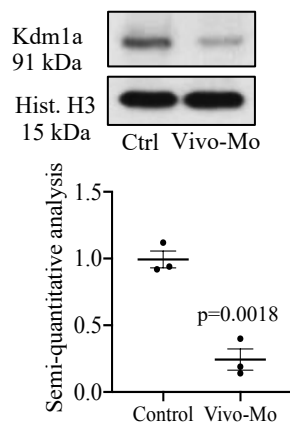
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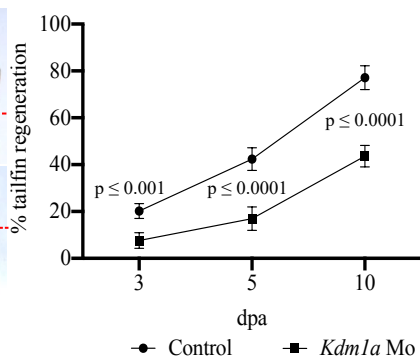
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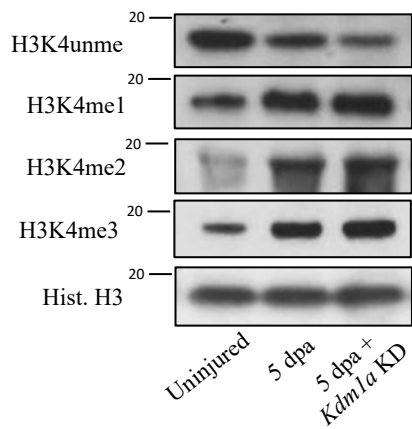
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